DIAGNOSIS OF EARLY MYOCARDIAL INFARCTION BY HISTOCHEMICAL STAINING OF HEART ON AUTOPSY TABLE

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BONAFIDE CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"DIAGNOSIS OF EARLY MYOCARDIAL INFARCTION BY HISTOCHEMICAL STAINING OF HEART ON AUTOPSY TABLE"** has been carried out by **Dr. K.Thunder Chief, M.B.B.S.,** a Post Graduate student under my supervision and guidance for his study leading to Branch XIV M.D. Degree in Forensic Medicine during the period of June 2009 to May 2012

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DECLARATION

I, Dr. K. Thunder Chief, solemnly declare that this dissertation titled **"DIAGNOSIS OF EARLY MYOCARDIAL INFARCTION BY HISTOCHEMICAL STAINING OF HEART ON AUTOPSY TABLE"** is the bonafide work done by me under the expert guidance and supervision of **Capt. Dr B. Santhakumar M.Sc., MD., DipNB(FM), P.G.D.M.L.E**, Director and Professor, Institute of Forensic Medicine, Madras Medical College, Chennai – 3. This dissertation is submitted to The Tamil Nadu Dr. M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIV) in Forensic Medicine.

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ABBREVIATIONS

AV - Atrio Ventricular

ECG - Electro Cardio Graph

FN - Fibronectin

HPE - Histo Pathological Examination

IHD - ischemic Heart Disease

IV - Inter Ventricular

LAD - Left Anterior Descending Artery

LV - Left Ventricle

MI - Myocardial Infarction

NAD - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

NADH - Nicotinamide Adenine Dinucleotide hydrogen

NBT - Nitro Blue Tetrazolium

RV - Right Ventricle

SA - Sino Atrial

TTC - Triphenyl Tetrazolium Chloride

WHO - World Health Organisation.

INTRODUCTION

INTRODUCTION

The incidence of Sudden Unexpected Death is increasing globally. Cardio vascular disease is the most common cause for sudden death. Eighty percent of sudden cardiac death is due to coronary arterial disease[36]. In 25% of cases death occurs abruptly and unexpectedly within first hour of onset of clinical symptom. Myocardial infarction has major psychological and legal implications for the individual and to the society. Establishment of clinical diagnosis of Myocardial Infarction usually becomes difficult as most often the death is sudden. Final chance to establish the cause of death is therefore the Post mortem Examination.

Determination of cause of death is the main objective in every Medico Legal Autopsy. Most often, the identification of early change of Myocardial infarction becomes difficult during postmortem examination, as gross change of infarct will not be apparent for 24 to 48 hrs following myocardial ischemic damage. "Identification of the earliest Stage of myocardial ischemia remains a pressing challenge"[41]. Histochemical staining of heart with azo dyes helps in identifying infarct areas from

normal myocardium. Histochemical staining techniques are based on the fact that ischemic myocardial cells lose their membrane integrity and release their enzyme contents into the blood, resulting in a marked decrease or total depletion of these enzymes in the ischemic areas of the myocardium. Enzyme depleted infarct myocardium remain unstained, which is the principle of this study.

Aims and Objectives

- To diagnose early Myocardial Infarction by Histochemical staining of Myocardium using Triphenyl Tetrazolium Chloride (TTC) while performing autopsy, in the absence of appreciable macroscopic changes in the myocardial tissue.
- To confirm those identified areas of early myocardial infarction by Histopathological Examination.
- To determine the diagnostic validity of the Histochemical Staining (TTC) of heart in gross detection of early Myocardial Infarction.

REVIEW OF THE LITERATURE

Review of the Literature

The situation of Forensic Pathologist performing Postmortem Examination on Sudden Natural Death cases is increasing, as most cases of sudden natural death comes under the scrutiny of investigating agencies. Identification of cause of death in such cases of sudden natural death helps in administration of justice, workmen's compensation claims, insurance benefits and provides medical data for framing health policies.

WHO defines sudden death as those that occurs within 24 hrs of onset of terminal illness. But no universally accepted standard time interval between onset of symptom to cessation of heartbeat and respiration defines death as "sudden death". Eighty percent of sudden cardiac deaths are due to coronary atherosclerosis. Identification of early myocardial infarction during autopsy pose difficulty, as apparent gross change of infarction takes 24 to 48 hrs following occlusion of major coronary artery in human.

Additional insight may be obtained from history, environment and circumstances of death. Even though minimal microscopic evidences are recognized as early as 6 hrs, in the absence of gross changes, the involved area may be missed when random blocks are taken for Histopathological examination. Histochemical staining of heart has thrown light into the diagnosis of early ischemic damage. Many Histochemical staining studies

are conducted on animal after inducing experimental infarction. Very few studies have been conducted on human heart during necropsy for detection of early myocardial infarction.

Pathology of acute Myocardial Infarction:

An understanding of Myocardial Infarction is based on the fact that the anatomy of coronary arteries is regional, i.e. each segment of myocardium has its own supply with little cross over with adjacent segments.

Anatomy of the coronary circulation:

Heart is a hollow muscular pumping organ situated in the middle mediastinum. Heart is supplied by right and left coronary arteries. The right coronary artery arises from the right Coronary Ostia at root of aorta, on leaving it passes anteriorly and travels in the coronary groove between atria and ventricle, and encircles the inferior border of the heart, where it gives marginal branch and runs in the posterior coronary groove where it gives posterior interventricular branch and that descends in the posterior interventricular groove towards apex (Fig.2). At the apex it anastomoses with the branches of left anterior descending artery. The marginal branch runs along the inferior border towards the apex.

The left coronary artery arises from left Coronary Ostia at aortic root, traverses smaller distance between left auricle and pulmonary trunk and reaches coronary groove, where it divides into left anterior descending artery and left circumflex artery. The left anterior descending (LAD) artery runs in the anterior interventricular groove towards the apex, where it anastomosis with the posterior interventricular artery (Fig.1).

The left circumflex artery lies in the coronary groove, encircles the left border of the heart and anastomosis with the terminal branches of the right coronary artery. In 33% of the individuals the posterior descending artery is the continuation of left circumflex artery [20]. Right coronary artery supplies right atrium, SA node, AV node, variable amount of left atrium, entire right ventricle except small area of anterior wall adjacent to the interventricular septum, posterior one third of the interventricular septum and small area of posterior wall of left ventricle adjacent to the interventricular septum.

Left coronary artery supplies left atrium, entire left ventricle except small area of posterior wall adjacent to the interventricular septum, anterior two third of interventricular septum and small area of anterior wall of right ventricle adjacent to the interventricular septum. By convention, the artery

which continues as posterior descending artery is called Dominant Artery. LAD constantly supplies 50 to 60% of total left ventricular mass.

Variations in the pattern of coronary artery and its branches and areas of the heart supplied by the respective coronaries are common. In many hearts, the left anterior descending artery is longer than the posterior descending artery, it encircles at the apex and supplies posterior wall of left ventricle[1]. Functional anastomosis or collateral blood flow between major arteries in normal heart is minimal, thus coronaries are referred to as "End Arteries". The weight of heart in males is 325 $\pm$ 75 grams, mean weight 300gms (0.45% of body weight), in females it is 275 $\pm$ 75 gms, mean weight is 250 gms (0.40% of body weight).[19]

Ischemic Heart Disease (IHD):

IHD is defined as acute or chronic form of cardiac disability resulting from imbalance between myocardial supply and demand for oxygenated blood. Coronary artery disease and Coronary heart disease are alternate terms for IHD as coronary artery narrowing or obstruction is the most common cause for myocardial ischemic injury. By the year 2020, it is estimated that IHD would be the most common cause of death throughout the world.[13] 90% of IHD is due atherosclerosis of coronary arteries, other cause accounts for less than 10% of cases of IHD. Ischemia represents

insufficiency of oxygen, reduced availability of nutrients and inadequate removal of metabolic end products.

Based on the rate of development, severity of narrowing of coronary artery lumen and myocardial response, IHD comprises of four clinical syndromes:

1) Angina pectoris,

2) Myocardial Infarction,

3) Chronic ischemic heart disease with failure,

4) Sudden cardiac death.

Myocardial Infarction (MI)

The term myocardial infarction reflects death of cardiac myocytes caused by ischemia, which is the result of a perfusion imbalance between supply and demand.[22] Myocardial infarction is the most important form of IHD, leading to death in most of the industrialized nations. Risk factors for Myocardial infarction include increasing age, hypertension, hypercholesterolemia, diabetes mellitus, hyperlipoprotenemia, cigarette smoking, obesity, hypertriglyceridemia, oral contraceptive pill (OCP) intake, sedentary life style, male gender, hypothyroidism,

hyperhomocsyteinemia, type 'A' personality and nephrotic syndrome. In the absence of predisposing atherogenic factors, women are protected from myocardial infarction during reproductive period of life. After menopause, women carry equal risk as that of men in development of MI. Hypertension is the stronger risk factor than hypercholesterolemia for MI after 45 yrs of age.

Pathologically myocardial infarction is classified into three categories as acute, healing and healed infarction. Acute myocardial infarction is characterized by infiltration of interstitium by polymorphonuclear leukocytes, if the time interval between the onset of the infarction and death is brief. The presence of mononuclear cells and fibroblasts, and the absence of polymorphonuclear leukocytes characterize healing infarction. Healed infarction is manifested as a scar tissue without cellular infiltration[22]. The entire process leading to a healed infarction usually takes at least 5–6 weeks[22].

Myocardial infarctions are classified based on size as,

1) Microscopic [focal necrosis],

2) Small [10% of the left ventricular (LV) myocardium],

3) Moderate [10–30% of the (LV) myocardium], and

4) Large [>30% of the (LV) myocardium].[22]

Pathogenesis of Myocardial Infarction:

Coronary arteries occlusion accounts for 90% of acute transmural myocardial infarction. Degree of lumen narrowing, length of stenotic segment and number of stenotic segment influences the development of myocardial ischemia. Any narrowing more than 75% of cross sectional area of the lumen of coronary artery increases the risk for development of myocardial ischemic damage. 90% of lumen occlusion produces impairment of coronary blood flow even at rest. Atheromatous plaques causing stenosis may be concentric or eccentric. In human, normal coronary vessel intima and media layers are avascular structures.

In the region of coronary artery affected by atheromatous plaque, new capillary from adventitia extends into media, breach inner elastic lamina and reach the intimal layer[40]. The intima of coronary arteries normally derives oxygen from the lumen which is impaired by atheromatous plaques. Hypoxia of the intimal layer of the coronary vessel is the strong stimulus for the ingrowth of the capillaries. Rupture of these small capillaries which enter the base of the plaque leads to extravasations of red cells into the plaque. This bleeding expands the plaque from within and potentially causes thrombosis. Large intraplaque thrombi contain large amount of fibrin and platelet in addition to extravasated red cells.

Hypotension during ischemic period further worsens the already precarious condition. Occlusive thrombosis of atherosclerotic coronary may occur without producing MI, owing to communicating vessel between major coronary trunks and collateral circulation. Pressure difference across the diseased vessel invokes collateral flow between normal vessel and the distal segment of the diseased vessel. Collateral blood flow in diseased heart is established by remodeling of existing vessels rather than by neogenesis[40].

In typical case of acute transmural MI, the following sequence of events takes place

1) Sudden gross change in the atheromatous plaque like ulceration, fissure or intra plaques hemorrhage.

2) Platelet adhesion, aggregation, activation release of factors on exposure to necrotic plaque and subendothelial collagen leads to formation of occlusive thrombus or emboli.

3) Released tissue thromboplastin initiates coagulation pathway.

4) Release of thromboxane A2, serotonin, and platelet factor favours vasospasm and increase the bulk of occlusive thrombus.

Within minutes the thrombus may evolve and occlude the lumen completely. Angiography after 12 to 24 hrs of onset of infarction reveals occlusive thrombus in only 60% of cases, because the thrombus may undergoes spontaneous lysis or relaxation of spasm[31]. The efficacy of thrombolytic therapy in restoring blood flow suggests that thrombosis is the major element in lumen narrowing.

In 10% of MI cases, coronary occlusion by emboli from mural thrombus, infective endocarditis vegetation, paradoxical emboli from right side of heart, dissection of coronary artery (common among women, LAD is commonly involved), vasculitis, hypovolumeic shock and coronary vasospasm plays causative role.

Origin of either coronary artery from pulmonary trunk has high risk of myocardial ischemia. The origin of both coronary arteries from one aortic sinus or single coronary orifice will not produce functional abnormality, unless the artery passes between pulmonary trunk and aorta[40]. One coronary orifice in the pulmonary trunk has higher serious sequelae. Epicardial arteries are covered by a layer of sub epicardial myocardium at certain points in varying degrees in up to 50% of cases. External compression of the arteries during systole by these bridging myocardial tissues may cause temporary occlusion or myocardial infarction[40].

Reperfusion of ischemic region may precipitate arrhythmias, myocardial hemorrhage with contraction bands and irreversible cell damage. Any discussion of reperfusion injury, although theoretically useful, is useless in human IHD as reperfusion injury has not been proven in any of the patient who has undergone reperfusion and vascularisation procedure by means of thrombolysis, angioplasty or coronary bypass graft[33].

Left Ventricle is most commonly affected. Most of the myocardial infarctions are transmural, involves full thickness of the wall of the ventricle in the region supplied by a coronary artery. In non-transmural infarct (sub endocardial infarction), the necrosis involves inner 1/3rd to ½ of the ventricular wall. It is more prone for ischemic damage, and has 20% greater oxygen utilization per gram of tissue. It also occurs in carbon monoxide poisoning and prolonged hypoglycemia. Diffuse sub endocardial infarction is common in end stage triple vessel disease, especially in diabetes mellitus and hypercholesterolemia.

Papillary muscle necrosis occurs in both transmural and sub endocardial infarction. The posterior medial papillary muscle supplied by right coronary is frequently affected than antero-lateral papillary muscle. Central zones of papillary muscle are the most vulnerable part of sub

endocardial tissue and ischemia damage occur first in that area[40]. In non transmural infarct, there is a lower incidence of total arterial occlusion. In case of profound hypotension, the decreased perfusion produces global infarction, and the myocardial damage is circumferential.

Multifocal microscopic foci of necrosis are of diverse origin. Their presence in the sub endocardial region represents an early stage of more confluent necrosis. Patients dying after prolong hypotension or hypoxia will have microscopic foci of necrosis. In severe left ventricular hypertrophy, microscopic foci of necrosis are common in the absence of impairment of coronary blood flow or hypoxia. High catecholamine both, iatrogenic and in phaeochromocytoma, ionotropic drugs leads to such foci of necrosis.

In all transmural infarction, at least a part of left ventricle including septum is involved. Isolated right ventricle infarct occurs in 1 to 3% of cases. Atrial infarct is usually associated with posterior left ventricular infarct. In 40 to 50 % of cases narrowing or thrombus occlusion occurs in left anterior descending artery, leading to infarct in anterior wall of left ventricle and anterior 2/3rd of IV septum. Right coronary artery occlusion occurs in 30 to 40 % of cases, produces infarct in posterior and inferior wall of left ventricle, posterior 1/3rd of IV septum and posterior

wall of right ventricle. Narrowing of left circumflex artery is less common (15 to 20 %), affects lateral wall of left ventricle. Thus occlusion of either coronary artery can cause left ventricular ischemic damage[5].

Thinning of the cardiac wall is always associated with an acute infarct affecting more than 30% of the left ventricular mass[33]. Extension of infarct may occur over a period of days to weeks. It is due retrograde propagation of thrombus, proximal vasospasm and impaired contractility of infarct zone that further compromises flow through stenosed artery. In extension of infarct, healing in the central region is advanced than the periphery of the infarct.

Morphology of myocardial infarction:

Before entering into actual morphology, let us have a brief look into relevant histology of myocardium: Cardiac muscle cell exhibits cross striations and the densely staining cross bands called intercalated disks. The intercalated disks represent highly specialized attachment between cells. This type of linear cell to cell attachment of cardiac cells results in "fibers" of variable length. Cardiac muscle consists of numerous cylindrical cells arranged end to end. Some cardiac muscle cells in a fiber

may join with two or more cells through intercalated disks, thus creating branched fiber[26] (Fig.3)

Intercalated disks contain two portions, transverse portion which runs across the fiber at right angle and lateral portion, which runs parallel to the myofilament. Gap junctions in the lateral portion of the disk provide ionic connectivity between adjacent cells. Each cardiac muscle possesses only one or two centrally located pale staining nuclei. Cardiac muscle cells contain numerous mitochondria, which occupy 40% of volume of cytoplasm, compared to 2% in skeletal muscle, reflect the need for continuous aerobic metabolism in heart muscle[24]. Lipofuscin pigment granules are found near the nuclear poles of cardiac muscle.

The location, size and morphological features of acute MI depend on rate of development, severity and duration of coronary occlusion, extent of collateral circulation present, site and severity of coronary spasm and metabolic demand of the myocardium at risk. Histologic detection of early infarct is difficult for pathologist, especially when death occurs within few minutes to hrs following an ischemic insult.

Gross morphology:

The gross morphological changes in the myocardium depend on the duration of patient survival following coronary occlusion.

Time	**Gross morphological features**
0 to 30 min	Nil change
½ to 4hrs	Nil change
4 to 12 hrs	Dark mottling (occasional)
12 to 24 hrs	Dark mottling in most cases
1 to 3days	Mottling with yellow tan infarct center
4 to 7days	Hyperemic border, central yellow tan softening
7 to 10 days	Maximally yellow tan, soft with depressed red tan margins
10 to 14 days	Red gray depressed infarct border
2 to 8 weeks	Gray white scar, progress from periphery towards center.
>2 months	Scarring complete.

Microscopic features of MI:

The microscopic appearances of human myocardial infarcts are complex.

Time	Light Microscope features
0 to 30 min	Nil changes
30 min to 4 hrs	Usually no change; variable waviness of fibers at the border
4 to 12 hrs	Early coagulation necrosis; edema; hemorrhage
12 to 24 hrs	Continuing coagulative necrosis; pyknosis of nuclei; myocytes hypereosinophilia; marginal contraction band necrosis; early neutrophils infiltration.
1 to 3 days	Coagulation necrosis with loss of nuclei and striations; brisk neutrophils infiltration.
3 to7 days	Beginning of disintegration of dead myofibers with dying neutrophils; early phagocytosis of dead cells by macrophages at the border.
7 to 10 days	Well developed phagocytosis of dead cells; early formation of granulation tissue at margins
10 to 14 days	Well established granulation tissue with new blood vessels and collagen deposition
2 to 8 weeks	Increased collagen deposition with decreased cellularity
>2 months	Dense collagen scar

Coagulation necrosis of myocardial tissue is the typical appearance in well established transmural infarct. Frequently, sub endocardial infarct is made up of coalescence of foci of necrosis of different ages.

Ultrastructural changes that occur during early phase of ischemic injury are 1) Glycogen depletion begins in 1st minute of occlusion and almost nil after 40 minutes. It is the first morphological evidence of ischemia[2] 2) Mitochondrial swelling occurs at 12 to 15 min, disintegration of cristae and vacuolization occurs by 30 min and membrane rupture occurs at 5hrs 3) Nuclei show chromatin clumping at 15 min and their membrane rupture after 3 to 4 hrs. 4) Stretching of myocells occurs within 15 to 30 min 5) Swelling of sarcotubular structure occurs by 30 min, myofibrils are relatively resistant and sarcolemma may rupture within 5 hrs[33].

Biochemical changes: Within seconds following coronary occlusion myocardial oxygen content falls and aerobic glycolysis stops. Creatinine phosphate level falls to zero in 2 to 3minutes. ATP begins to fall after2 to 3min[2].

Waviness of fibers:

It is the deformation of myocardial fiber that occurs during early phase of infarct development, in cases, in which death has occurred

suddenly or delayed for 2 to 3 hrs. The ischemic fiber are rhythmically bent as well as stretched and the waves are formed by adjacent fiber. There are smaller waves in the spectrum which lead to classification of waviness of myocardial fibers into three orders.

First order waves: These are small wiggles formed by single myocardial fiber. The twisted fiber occurs singly as aberration in a patch of otherwise normal myocardium or sometimes along the second order waves.

Second order waves: Here bundles of fibers are affected; the length of the wave is about 0.3mm and remains fairly constant within given focus.

Third order waves: It occurs when bundle of twisted fiber is in turn undulating and it is least common. Waving of myocardial fibers is evident, on cutting the fibers longitudinally. If cross sections are made banded pattern will result, appears as band of segments (crest or sides of the waves) alternating with band of dots (cross section of fiber). Therefore diagnosis of Waviness of fiber is possible even with cross section.

Most 'typical patch' of wavy myocardium is of second order waves. Usually evident at the edges of infarct, never extend throughout the infarct unless the infarct is very small one. The transition between normal myocardium and wavy fiber is sharp. Sub endocardial region is usually

spared from waviness as it is supplied by diffusion from endocardium. Waviness of fibers in the sub endocardial region occurs when the endocardium is pathologically thickened or covered with thrombus.

Pathogenesis of waviness of fibers:

If some fiber in the bundle of straight parallel fibers becomes wavy and thinner, it is logic to consider the fiber has been stretched and elongated too long for the bundle. Acute ischemia of myocardium will stop the contractile function of fibers in 60 sec[31]. These immobilized fibers are in attachment with the normal contracting myocardial fibers at both ends, therefore at every systole the ischemic fibers are subjected to powerful tug, resulting in thinning and elongation. The fibers are too long than the stroma which contains them, resulting in forced bending of fibers into wave pattern.

Second mechanism is that the entire thickness of infarcted myocardium begins to bulge at every systole under the influence of intracardiac pressure, resulting in loss of elasticity to the ventricular wall. Wavy fiber pulls back to its normal shape and length i.e. reversible. If the ischemia continues until polymorphs infiltrates then the wavy fibers are probably dead.

Contraction bands:

The term "Contraction bands" was first introduced by Caulfield and Klionsky in 1959. It is dense eosinophilic transverse band within the muscle cell, represents telescoped sarcomeres and a form of pathological shortening. It is a useful adjunct in the diagnosis of early myocardial infarction (up to 6hrs).[4] It appears in a narrow zone of myocardium between wavy fiber and normal tissue. Calcium ions flooding into the cell, evoking focal hypercontraction of adjacent viable tissue and shunting together of sarcomeres results in the formation of "contraction band". Histologically contraction band necrosis may be recognizable within 20 min of onset of infarction in contrast to coagulative necrosis, which needs 4 to 6 hrs survival for recognization.

Polymorphs infiltration: In 5 to 6 hrs after occlusion of coronary vessels, polymorphs marginate within the vessels at the periphery of the affected area and penetrate centripetally into the necrotic tissue[33].

Vascular changes: The constant findings are dilatation and congestion of venules and capillaries.

Clinical features:

Signs and symptoms include chest pain, rapid thin pulse, profuse sweating and dypsonea due to pulmonary congestion and edema. Silent

myocardial infarction occurs commonly in elderly and diabetic patients due to neuropathy of afferent fibers that conducts pain. Diagnosis of Myocardial infarction is made from clinical symptoms, Electrocardiographic changes and detection of myocardial proteins and enzymes in the serum.

Complications:

Post-myocardial infarction complications include Cardiogenic shock (major cause of mortality in patients whose infarcts involves more than 40% of total left ventricular mass)[40], Ventricular tachy-arrhythmias, Dressler's syndrome, myocardial free wall rupture produces electromechanical dissociation, cardiac tamponade and rapid death, free wall rupture is common in females, age over 60 yrs and preexisting hypertension, rupture of interventricular septum results in ventricular septal defect and left to right shunt, infarct extension and expansion, pericarditis, development of mural thrombus, papillary muscle dysfunction and ruptures (rupture of posterior papillary muscle is 4 to 7 times more frequent than antero- lateral papillary muscle rupture) cardiac failure, and ventricular aneurysm.

Patients with MI, who are immobilized for long period, have higher risk of developing deep venous thrombosis and possibility of pulmonary

embolism. Early mobilization reduces incidence of post infarction pulmonary embolism[18]. Sub endocardial infarction has good initial prognosis, incidence of cardiac failure and death is less compared to transmural infarction. But the risk of subsequent arrhythmias and reinfarction is higher with sub endocardial infarction[18].

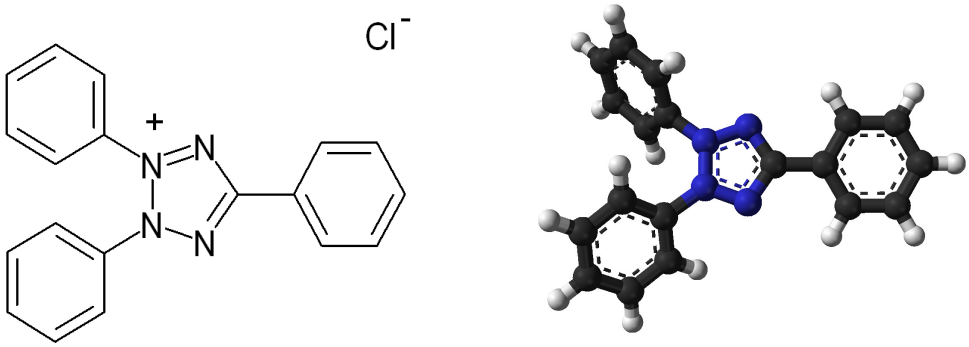
Methods for detection of early myocardial infarction in autopsy cases:

Various methods have been discovered for identification of early myocardial infarction during autopsy. Each method has its own advantages and limitations. Many animal experiments were conducted using various methods for detection of early infarction. In the year 1960, Histochemical staining using Tetrazolium dyes was introduced as a method to identify early MI. Other methods include measuring K+/Na+ ion ratio, mg+/Ca+ ion ratio, Hematoxylin-basic fuchsin-picric acid staining, Barbeito-Lopez Trichrome staining, fluorescent microscopy, measurement of sarcomeres length and determination of density of blood.

Later immunohistochemical methods to detect loss of glycogen, myoglobulin, intracellular diffusion of IgG, fibrinogen complement C5b-9, caeruloplasmin, C-reactive protein, troponin T, cytoskeleton proteins

vinculin, desmin, alpha-actinin and fibronectin for identification of early MI were discovered. Immunohistochemical methods carry greater advantage as the technique was not affected by postmortem autolysis and formalin fixation. It can be used for retrospective analysis of cases. The limitation of the method is the high cost of the marker. Histochemical staining using Tetrazolium salts are simpler, reliable and cost effective method for detecting early MI. Two azo salts are Triphenyl Tetrazolium chloride (TTC) and Nitroblue Tetrazolium (NBT). Advantage of TTC are low cost Rs 200, compared to NBT which costs Rs 5000, TTC penetrates cell membrane whereas NBT will not penetrate cell membrane[17].

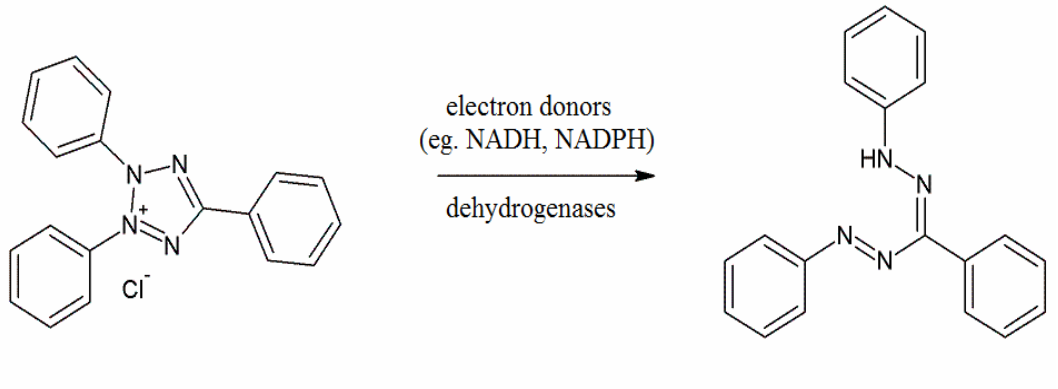
Triphenyl Tetrazolium Chloride ($C_{19}H_{15}ClN_4$)



Structure of 2, 3, 5 Triphenyl Tetrazolium Chloride

Triphenyl Tetrazolium Chloride, **TTC**, or simply **Tetrazolium chloride** is a redox indicator commonly used in biochemical

experiments especially to differentiate metabolically active and inactive tissues. It is a white crystalline powder, and the molecular weight is 334.8 grams. It is soluble in water, ethanol and acetone but insoluble in ether. Chemically Tetrazolium salts have ring structure which consists of one carbon and four nitrogen atoms, of which one is quaternary. Tetrazoles are converted into colored formazans on uptake of one proton and two electrons.



TTC (White compound) TPF (Red compound)
1,3,5 Triphenyl Formazan

The tetrazoles salts are not dyes in fact, but on reduction they form colored water-insoluble pigments known as formazans. Reduction of Tetrazole salts are associated with opening of ring leading to formation of colored formazans. Usually white Tetrazolium salts are reduced by redoxsystem which has low redoxpotential than the dye itself. Most important biological redoxsystem are NAD / NADH, NADP / NADPH,

cytochrome b, cytochrome b5 cytochrome p450. On oxidation of NADH and NADPH by dehydrogenases; free electrons are picked up by the tetrazoles, which becomes reduced.

At the stage of ischemic damage there is marked decrease in the level of enzyme such as dehydrogenase, diaphorases and coenzymes. The infarct areas will be depleted of above enzymes and remain unstained. The formation of colored Formazan is a surface phenomenon, and it does not affect subsequent Hematoxylin and eosin staining of the sections. TTC is heat and light unstable molecule so, these environments should be avoided as much as possible.

Some of the studies which were conducted earlier to detect early myocardial infarction using various methods are as follows:

Theodar K Shnikta et al in 1963: They produced Experimental myocardial infarct in 46 dogs by high ligation of the anterior descending branch of the left coronary artery. Dogs were euthanized at different time interval. Histochemical and morphologic alterations were observed at intervals from 1hour to two and half weeks. They showed that the earliest loss of respiratory enzyme activity was recognized at about 6 hours[38].

Ramkissoon RA in 1966 used Nitro BT to demonstrate early myocardial infarctions on 31 human hearts in cases of sudden death and

severe congestive heart failure with acute coronary insufficiency. The outcome of the study shows, earliest myocardial infarct to show loss of dehydrogenase enzyme activity was of eight hrs duration[30].

McVie in 1970 compared potassium / sodium ratio in infarct heart tissue with Tetrazolium staining of heart for detecting inapparent myocardial infarction. He suggested that macroscopic Tetrazolium staining is useful screening test for early inapparent myocardial infarction, but the ionic ratio method is not affected by autolysis and well suited for forensic practice[25].

Feldman S et al 1975 perfused heart with Nitroblue Tetrazolium solution and subsequent with 3.7% of formaldehyde solution. They observed for three dimensional infarct distributions[10].

Rammer L et al in 1976 compared potassium / sodium ratio in the myocardium with the result of PTAH (phosphotungstic acid Hematoxylin) staining method and they found electrolyte method appears to be more sensitive method for diagnosis of recent myocardial infarction[29].

Decastello A et al in 1977 determined K+/ Na+ ratio in myocardial specimens from 90 postmortem cases. They suggested determination of myocardial K+/Na+ ratio provides great aid in detecting early myocardial infarction. The technique is not affected by autolysis[7].

Derias NW et al in 1978, stained heart from 81 cases of suspected myocardial infarction using Nitroblue Tetrazolium and suggested, the method may be of diagnostic value at necropsy from one hour onwards after the time of clinical onset of infarction.[8]

Fishbien MC et al in 1981, conducted study to assess the accuracy off TTC Histochemical technique for quantitation of early myocardial infarct size in 16 closed chest dogs. They concluded TTC technique represents a reliable and practicable method of quatitation of recent infarction (6 hrs and even 3hrs after occlusion of coronary artery) and for studying the evolution of ischemic injury in its early stage[11].

Kloner RA et al in 1981 studied the accuracy of TTC staining of heart in delineating early ischemic myocardial injury prior to appearance of well defined histologic necrosis.

The study was carried out on 8 dogs, ultrastructure of the TTC unstained areas under electron microscopy showed numerous mitochondrial amorphous dense bodies, intracellular and interstitial edema, nuclear clumping and margination and sarcolemma disruption. They suggested TTC is a reliable stain for delineating myocardial tissue that ultrastructurally appears irreversibly injured by ischemia[21].

Milei et al in 1983, compared Barbeito-Lopez Trichrome stain with H-E, basic fuchsin picric acid and Nitroblue Tetrazolium stain. They

reported Barbeito-Lopez Trichrome was more sensitive than the other stains for the diagnosis of early myocardial coagulation necrosis[27].

Fujita M et al in 1985, studied diagnosis of early myocardial infarction using Hematoxylin basic fuchsin picric acid (HBFP) in 42 human hearts. Diagnosed MI cases, sudden death cases from post infarct angina stained HBFP positive. Sudden death cases due to non ischemic, non cardiac and arrhythmias were stained HBFP negative. They concluded HBFP staining method is a reliable method for identifying early myocardial ischemia and extent of early infarction[12].

Shperling ID in 1986, he measured sarcomeres in the infarct area in sudden death, outside infarct zone and without acute infarction in the myocardial tissue of patients with IHD and in patients died of other causes. The study showed, sarcomeres relaxation occurs in extensive areas of muscle fiber in the early stage of infarction, and it is not seen outside the area of acute myocardial ischemic damage[35].

Badir B et al in 1987, studied detection of early myocardial infarction using Fluorescent microscopy. The sections are specifically stained by fluorochrome dyes. They said the method has advantage of sensitivity, simplicity and speed compared with complex enzyme hitsochemistry and electron microscopy[3].

Chopra P et al in 1988, they produced experimental myocardial infarction in rats by ligating left coronary artery for 5min to 6 hrs. Fluorescent techniques and Tetrazolium staining of the hearts were done to identify, a sensitive technique for use in routine pathological specimens. They found Triphenyl Tetrazolium and Nitroblue Tetrazolium staining of myocardium showed loss of dehydrogenases within 5 to 20 min of ligation of the artery. Auto fluorescence in formalin fixed, Hematoxylin and eosin stained sections showed positive fluorescence only after 50 to 75 min of ischemia. Examination of myocardium under fluorescent light after staining with Acridine orange is more sensitive than autofluorescence for detecting ischemia[6].

Varga M et al in 1988, conducted analysis of K+/Na+ ion ratio using flame spectrometer after decomposition of myocardium by Lumatom tissue solubizer. They found ion content and their ratios correlate well with ECG, clinical data, the autopsy findings and histological changes[39].

Lachica E et al in 1988 conducted studies in Granada, Spain using four methods Eosin-Hematoxylin, Acridine Orange method, Formazans test and K+/Na+ ratio for comparing the reliability of method in detecting early myocardial infarction. Although Formazan and K+/Na+ ratio showed good reliability to rule out MI, the most specific technique for detection of infarction is the Formazan test[23].

Hougen HP et al in 1992 studied Histochemical, Biochemical and Morphological methods for detection of myocardial infarction in 150 cases in Copenhagen, Denmark. The result showed combination of different methods leads to diagnosis of myocardial infarction in far more cases than with morphological or biochemical methods alone[15].

Doran JP et al in 1996, conducted immunohistochemical staining for complement C9 as a method of detection of early myocardial infarction and found the method has higher sensitivity for detection of early myocardial necrosis. The method is simple, reliable and could be done on formalin fixed, paraffin embedded heart specimens[9].

Hu Bj et al in 1996, they conducted immunohistochemical study of fibronectin (FN) on 34 cases. 23 cases of definite and suspected myocardial infarction showed positive staining, no staining in 11 non cardiac controls. In 5 cases cardiac tissue was fixed in 10% formalin for 10 yrs and examined, FN immunohistochemistry yield satisfactory result in these 5 cases. The study concluded FN was not affected by postmortem autolysis and formalin fixation, could be used in routine autopsy practice, particularly in retrospective analysis of cases[16].

Adegboyega et al in 1997 stained 638 hearts from cases suspected or diagnosed MI using TTC dye. They concluded that, the Histochemical

detection of grossly inapparent MIs with TTC is a useful adjunct in the diagnosis of acute myocardial ischemia[1].

Mortensen ES et al in 2008 determined the frequency of acute myocardial infarction and estimation of infarct age by changes in the hitsomorphological characteristics in cases of sudden death (15 cases due to coronary heart disease and 15 cases due to non cardiac cause). They observed that majority of cases in the coronary heart disease group died with extensive symptomatic myocardial infarction[28].

Rzepecka Woniak E. et al in 2008, researched at introducing the immunohistochemical C9 staining technique as a postmortem diagnostic method of detecting recent myocardial infarction for the purpose of postmortem medico legal examination. The results are compared with the result of Nielsen- selye staining method. The study demonstrated that immunohistochemical C9 staining technique has higher specificity and usefulness in detecting recent stage of myocardial infarction[32].

Jie Quyang et al in 2010 studied 25 human hearts to investigate the utility of Masson's trichrome, and immunohistochemical markers for the myocyte protein desmin, in detection of acute myocardial infarction and ischemia. They suggested that desmin and Masson's trichrome are valuable tools, when faced with the question of early myocardial

ischemia/infarction in autopsy material. The postmortem interval did not adversely affect the histochemical changes or immunohistochemical in this study[19].

M Shankar Bakkannavar et al in 2011 conducted Histochemical staining of heart using TTC in 40 cases of sudden death. The study confirms the usefulness of staining in detecting infarct in suspected sudden death cases[3]

MATERIALS AND METHODS

MATERIAL AND METHODS

The present study was conducted in the Institute of Forensic Medicine in collaboration with Institute of Pathology and Electron Microscopy, Madras Medical College, and Department of Chemistry, College of Pharmacy Chennai-3 for period of one year. The study sample consists of 18 hearts taken from cases of sudden deaths with either history or the morphological features of heart suggestive or suspected the cause of death to be of cardiac origin. Before getting into the study, Ethical clearance was obtained from Ethical committee headed by the chairman.

During Postmortem examination heart was removed, washed thoroughly under running water and weighed. Gross examination of the entire heart was done to look for any presence of scar due to old infarct, areas of softening surrounded by hyperemia or any other morbid condition. Serial sections of coronary artery were made at the distance of 3mm to look for any presence of occlusion by plaques or thrombus. The consistency of the coronaries was appreciated. Serial transverse section involving full thickness of heart was made at the distance of 1 cm each from the apex to the AV groove. Slices are examined for old fibrotic scar and softening. The heart is dissected along line of flow of blood, observed for raised atheromatous plaques on the luminal surface of root of aorta,

narrowing of coronary Ostia, and narrowing of lumen of coronaries by atheromatous plaque or thrombus. Two slices are selected at random between mid ventricle and apex is taken for histochemical staining.

Preparation of TTC Solution:

Chemicals required are

1) Sodium Dihydrogen Phosphate (NaH2Po4) - low pH phosphate buffer (Fig. 5). Its molecular weight is 120 grams.

2) Disodium hydrogen phosphate (Na2HPo4) – high pH phosphate buffer (Fig.4). Its molecular weight is 142 grams.

3) Distilled water (Fig. 6).

4) Dye - Triphenyl Tetrazolium Chloride (TTC) (Fig.7)

12 grams of sodium Dihydrogen phosphate is dissolved in one litre of distilled water to make 0.1 M solution. 14.2 grams of Disodium hydrogen phosphate is dissolved in one litre of distilled water to make 0.1 M solution[17]. pH of the solution was adjusted by mixing 0.1M Disodium hydrogen phosphate solution and 0.1M sodium Dihydrogen phosphate in different proportion as follows:

pH	0.1M Na2HPO4 solution, (High pH)	0.1M NaH2PO4 solution, Low pH (%)
5.8	079 ml	921 ml
6.0	120 ml	880 ml
6.2	178 ml	822 ml
6.4	255 ml	745 ml
6.6	355 ml	645 ml
6.8	463 ml	537 ml
7.0	577 ml	423 ml
7.2	684 ml	316 ml
7.4	774 ml	226 ml
7.6	845 ml	155 ml
7.8	**896 ml**	**104 ml**
8.0	932 ml	68 ml

896 ml of 0.1M sodium Dihydrogen phosphate solution and 104 ml of 0.1 M Disodium hydrogen phosphate solution was mixed up to attain pH of 7.8. pH of the buffer solution was confirmed using pH meter. Ten grams

of Triphenyl Tetrazolium Chloride was then dissolved in one litre of above phosphate buffer solution of pH 7.8 to make 1% TTC solution[17]. 1% TTC solution was stored in Amber colored bottle (Fig.8) as the dye TTC is photosensitive, gets inactivated on exposure to light.

Staining method:

Heart slices were washed with running water and wiped with tissue paper and placed in a plastic container of size larger than the heart slice (Fig.10). One container was used for each slice. TTC solution was poured into the container containing heart slice, so that the solution level in the container was about 2 cm above the heart slice to prevent atmospheric oxygen penetration. Then the system is placed in a cardboard board box to avoid light exposure. The incubation is carried out at room temperature for 20 minutes. Heart slices should not touch the container surface as it may result in artifactual nonstaining. Ten minutes after incubation, the heart slice was turned upside down to prevent artifactual nonstaining.

After incubation for 20 minutes, heart slices were removed and examined for unstained areas of myocardium. Normal myocardium stained brick red whereas infarct area remained unstained or shows very much reduced staining. The positive result was inferred by suspected infarcted

area remaining unstained with the TTC. Both the stained and unstained areas were subjected for paraffin embedded histological examination. In TTC positive cases, sections were taken from unstained area, whereas in negative cases two random sections were made from the left ventricle. Histopathological diagnosis of acute myocardial infarcts is made from following criteria, which include wavy myofibers, coagulative necrosis, ischemic contraction band necrosis, myonecrosis, and polymorphs infiltrations of the interstitium[1]. Then the results of TTC staining were correlated with the Histopathological examination findings.

MASTER CHART

TABLE: 1

S.No	PM.NO:	AGE	SEX	TIME SINCE DEATH(APPROXMATE)	GROSS APPEARANCE OF HEART	GROSS APPEARANCE OF HEART SLICE BEFORE STAINING	GROSS APPEARANCE OF HEART SLICE AFTER STAINING	HPE FINDINGS
1	1690/11	72	M	8 hrs	Enlarged in size, weighs 610 grams aortic valve calcified and narrowed, left ventricle wall thickness measures 1.7 cm, multiple atheromatous plagues on root of aorta, left anterior descending artery, proximal RC thickened and narrowed.	Old whitish grey fibrotic tissue in the anterior wall of distal LV and adjoining interventricular septum	Anterior wall and adjoining IV septum unstained and posterior wall of left ventricle and small area of right ventricle unstained	Section from unstained area shows extensive old infarct and fresh infarct with dense infiltration of polymorphs with Pyknotic nuclei and wavy fibers. Section from stained area showed normal myocardial histology.
2	1794/11	80	M	15 hrs	Enlarged in size, weighs 560 grams. Multiple atheromatous plagues on inner side aortic root. Left ventricle thickness measures 1.7 cm. proximal 2 to 3 cm of arteries LAD, LC and RC were thickened calcified and narrowed.	Old fibrotic scar tissue in the posterior wall of LV and right ventricle.	The posterior wall of LV and right ventricle remain unstained.	Section from unstained area shows myocardium with multiple foci of infarction showing hyalinised collagen fibers. Section from stained area showed normal histology.
					Enlarged in size, weighs 600 grams. Multiple	No abnormality	Areas of pale	Section from pale stained

3	1850/11	50	M	19hrs	atheromatous plagues on inner side aortic root. Left ventricle thickness measures 2 cm. left anterior descending artery thickened and lumen narrowed.	detected.	staining in the anterior wall of left ventricle near the apex region.	area shows infarct with minimal infiltration of neutrophils. Section from stained area showed normal myocardial histology.
4	1916/11	34	M	16 hrs	Normal in size, weighs 260 grams multiple atheromatous plagues on root of aorta. All three vessels were thickened, narrowed and shows focal calcification.	No abnormality detected	Area of unstaining in the anterior wall near apex region	Section from unstained area shows myocardium with normal histology.
5	1921/11	71	M	16 hrs	Enlarged in size, weighs 460 grams. Multiple atheromatous plagues on root of aorta. All three vessels were thickened, calcified and narrowed.	No abnormality detected.	All area of myocardium stained, no unstained area	Section from heart showed myocardium with normal histology, no infarct made out.
6	1972/11	70	M	30hrs	Normal in size, weighs 270 grams, multiple atheromatous plaques on inner surface of aortic root. proximal part of all three coronaries were thickened and narrowed	Posterior wall of the left ventricle shows old fibrotic scar.	Posterior wall of the left ventricle and patchy area in the sub endocardial region of the antero-lateral wall and IV septum remain unstained	Section from unstained area shows evidence of old infarct with scarring in some foci and focal area of fresh infarct with diffuse infiltration of polymorphs, Pyknotic nuclei of myocardial fibers and increased eosinophilia. Section from stained area showed normal histology.

7	2073/11	65	M	27hrs	Enlarged in size, weighs 570 grams. Multiple atheromatous plaques on inner surface of aortic root. Proximal 2 to 3 cm of all three coronary vessels were thickened, narrowed and calcified Diffuse hyperemic patch with softening seen in antero lateral wall of the left mid ventricle region.	Antero lateral wall of the left mid ventricle shows patchy softening and hyperemia and old fibrotic scar in the posterior wall of LV and RV	Anterior and lateral wall of the LV unstained and area of unstaining of the posterior wall of LV and RV.	Section from unstained area shows focal areas of dilated and congested blood vessel, foci of myocardial fibers with intense eosinophilia, Pyknotic nuclei with infiltration of neutrophils and wavy fibers. Some foci show evidence of old infarct. Section from stained area showed normal histology.
8	2136/11	50	M	5 hrs	Heart was normal in size, weighs 280 grams.Multiple atheromatous plaques on inner surface of aortic root. Left anterior descending artery wall thickened and the lumen was occluded with thrombus	No abnormality detected.	Anterio lateral wall and adjoining IV septum of left ventricle remain unstained.	Section from stained areas shows normal histology. Section from unstained area shows focal collection of polymorphs, nucleomegaly with adjoining wavy fibers and congested blood vessels. Some of the myocardial cells show Pyknotic nuclei.
9	2144/11	59	M	5 hrs	Enlarged in size, weighs 470 grams. Multiple atheromatous plagues on root of aorta. Left ventricle thickness measures 1.9 cm. Proximal part of all three	Anterior wall of left ventricle and adjoining interventricular septum and lateral wall of LV shows	Unstaining of the anterio-lateral wall of left ventricle and anterior septum.	Section from unstained portion of heart slice reveals areas of myocardial infarction with myocardial fibers replaced by dense band of fibrocollagenous tissue. Section from stained

					coronary vessels was thickened narrowed with calcification of LAD.	fibrotic old scar tissue		area showed normal myocardial histology.
10	2216/11	58	M	24 hrs	Enlarged in size weighs 480 grams. Old infarct scar on the posterior wall of the left ventricle. LV thickness 1.9 cm Multiple atheromatous plagues on root of aorta. Narrowing of coronary Ostia. Proximal part of all three coronary vessels was thickened narrowed and calcified.	Old infarct fibrotic scar in the posterior wall of the LV.	Posterior wall and sub endocardial tissue in the lateral wall of the left ventricle remain unstained.	Section from unstained area shows wavy fibers, intense eosinophilic cytoplasm and pyknosis of nucleus. No polymorphs made out. Some areas myocardium is replaced by dense collagenous tissue. Section from stained area revealed normal histology.
11	2259/11	51	M	21hrs	Enlarged in size, weighs 510 grams multiple atheromatous plagues on inner surface of aortic root. Proximal part of the all three coronary vessels were thickened, narrowed and calcified.	No abnormality detected.	All regions of the ventricles stained.	Section from the stained area of heart slice shows normal myocardial fibers
12	2496/11	44	M	26 hrs	Normal in size, weighs 280 grams, multiple atheromatous plaques on inner surface of aortic root and coronary vessel wall. Lumen of the coronary vessels was narrowed.	No abnormality detected.	All area of the myocardium stained.	Section from the stained area shows focal areas of wavy fibers, nucleomegaly and mild infiltration of polymorphs.

13	2513/11	72	M	23 hrs	Heart was enlarged in size, weighs 560 grams LV thickness was 1.9 cm, multiple atheromatous plaques on inner surface of aortic root, coronary ostia narrowed, and all three coronary vessels were thickened narrowed and calcified.	Irregular patchy fibrotic scar tissue in the posterior wall of left ventricle and posterior wall of right ventricle.	Unstaining of the posterior wall of left and right ventricle. Irregular Unstaining of sub endocardial region antero-lateral wall of LV.	Section from pale area show features of myocardial infarction with myocytolysis and inflammatory cell infiltration. Foci of old infarct and fibrotic scar. Section from stained area showed normal histology.
14	2550/11	46	M	16 hrs	Heart was enlarged in size, weighs 460 grams LV thickness measures 1.8 cm, multiple atheromatous plaques on inner surface of aortic root. Proximal part of all three coronary vessels was narrowed, thickened.	No abnormality detected.	Entire myocardium stained	Section from the stained heart slice shows normal myocardium. No areas of infarct made out.
15	2570/11	56	M	17 hrs	Heart enlarged in size, weighs 510 grams multiple atheromatous plaques on inner surface of aortic root. Coronary ostia narrowed, left anterior descending artery and left circumflex artery were thickened, calcified and narrowed.	No abnormality detected	Unstaining of sub endocardial area of anterior, septal and posterior wall of left ventricle.	Section from unstained area shows myocardium with foci of infarction and necrosis of the myocardial fibers with early infiltration of neutrophils. Section from stained area shows normal myocardium.

16	2790/11	38	M	22 hrs	Enlarged in size, weighs 440 grams. Left ventricle thickness measures 1.8 cm, multiple raised atheromatous plaques on the inner surface of aortic root. Proximal part of left anterior descending thickened and lumen occluded with thrombus.	No abnormality detected	Anterior wall of left ventricle and adjoining IV septum near apex unstained	Section from unstained area shows wavy fibers, nucleomegaly and acute inflammatory cell infiltration. Section from stained area reveals normal histology.
17	2864/11	55	M	20 hrs	Enlarged in size, weighs 470 grams, left ventricle thickness measures 2 cm. Old fibrotic scar on the posterior wall of left ventricle. Multiple atheromatous plaques on the inner surface of aortic root. All three coronary vessels were thickened, narrowed and calcified.	Small area of whitish grey fibrotic old scar in the posterior wall of left ventricle.	Sub endocardial region of the entire left ventricle and posterior wall of the left ventricle unstained.	Sections from unstained areas shows foci of infarction with minimal neutrophils infiltration, and some foci shows evidence of old infarct. Section from stained area showed normal histology.
18	2909/11	70	F	26 hrs	Enlarged in size, weighs 360 grams, multiple atheromatous plaques on the root of aorta, proximal 3 to 4 cm of LAD thickened and narrowed	No abnormality detected	Areas of unstaining in anterior wall of left ventricle and posterior wall of left ventricle and right ventricle.	Section from the unstained area shows myonecrosis and minimal neutrophils infiltration. Section from the stained area shows normal myocardial histology.

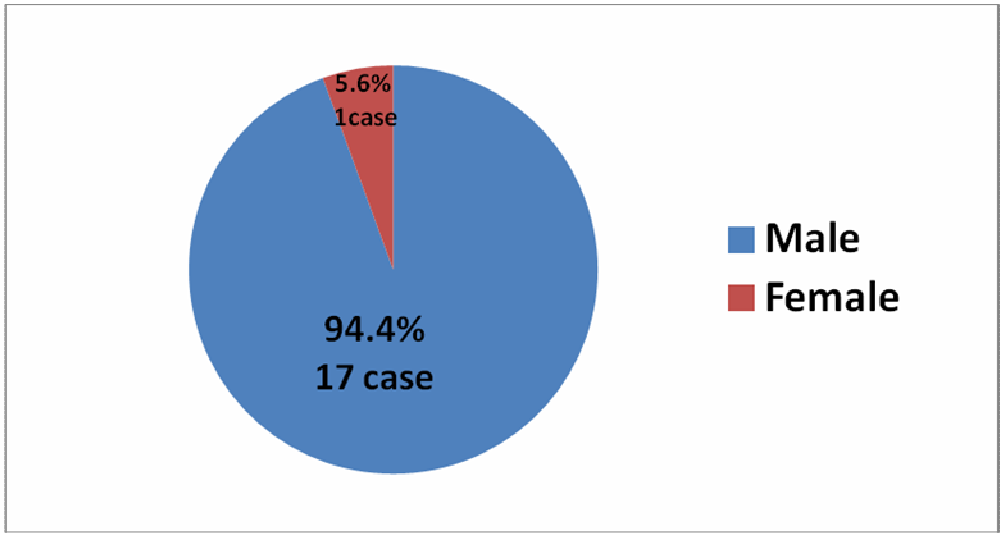
Analysis and Results

Analysis and Results

Table: 2 Sex distributions among the study sample.

Sex	Frequency	Percentage
Male	17	94.4%
Female	01	5.6%
Total	18	100%

Fig: 1 Sex distributions among study sample.



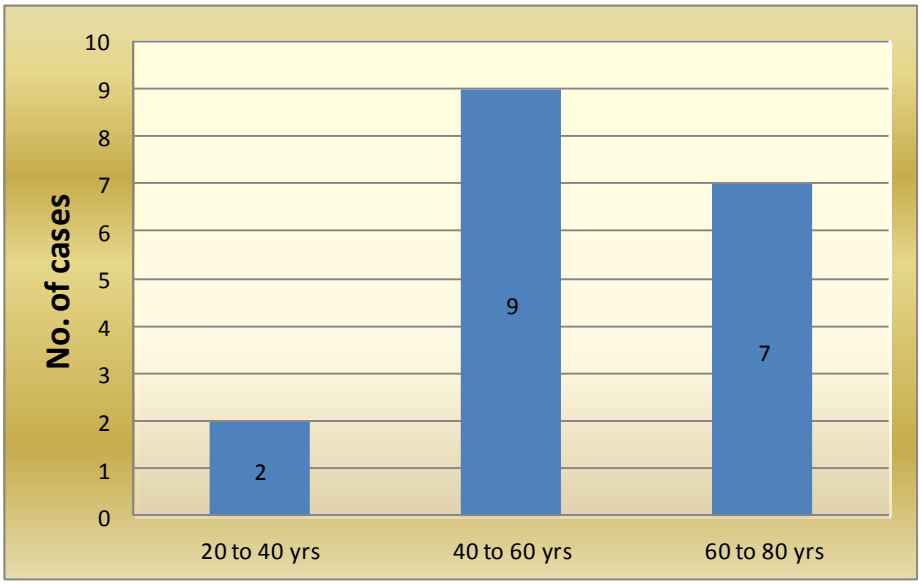
Males occupy predominant number of cases, accounts for about 94.4% of sample, whereas female constitute only 5.61% of the sample. It

signifies the incidence of myocardial infarction / sudden cardiac deaths are greater among males.

Table: 3 Age group wise distributions of the study sample:

Age distribution	No. of Cases	Percentage
20 to 40 yrs	**2**	**11.1%**
40 to 60 yrs	**9**	**50%**
60 to 80 yrs	**7**	**38.9%**

Age wise distribution chart:

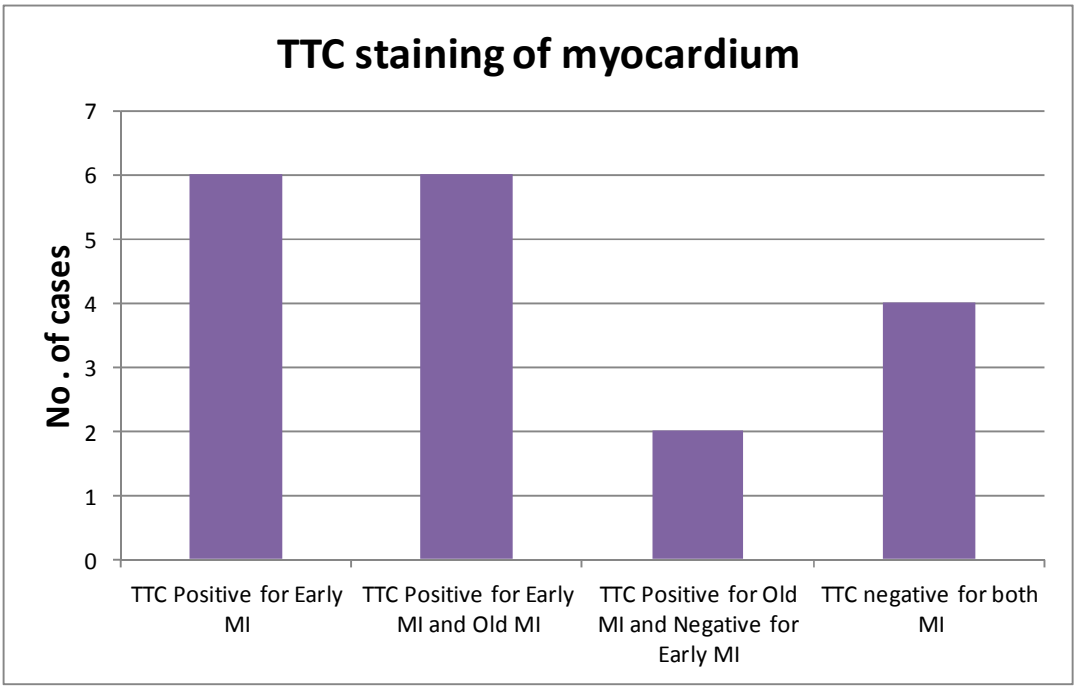


Cases in the age group 40 to 60 yrs accounts for about 50% of the sample cases, 38.9% cases are in the age group of 60 to 80 yrs. 2 cases in the age

group 20 to 40 yrs accounts for 11.1% of total cases. The least age in the study sample is 34yrs and the maximum age encountered in the study was 80 yrs.

TABLE: 4 TTC STAINING FOR EARLY MYOCARDIAL INFARCTION:

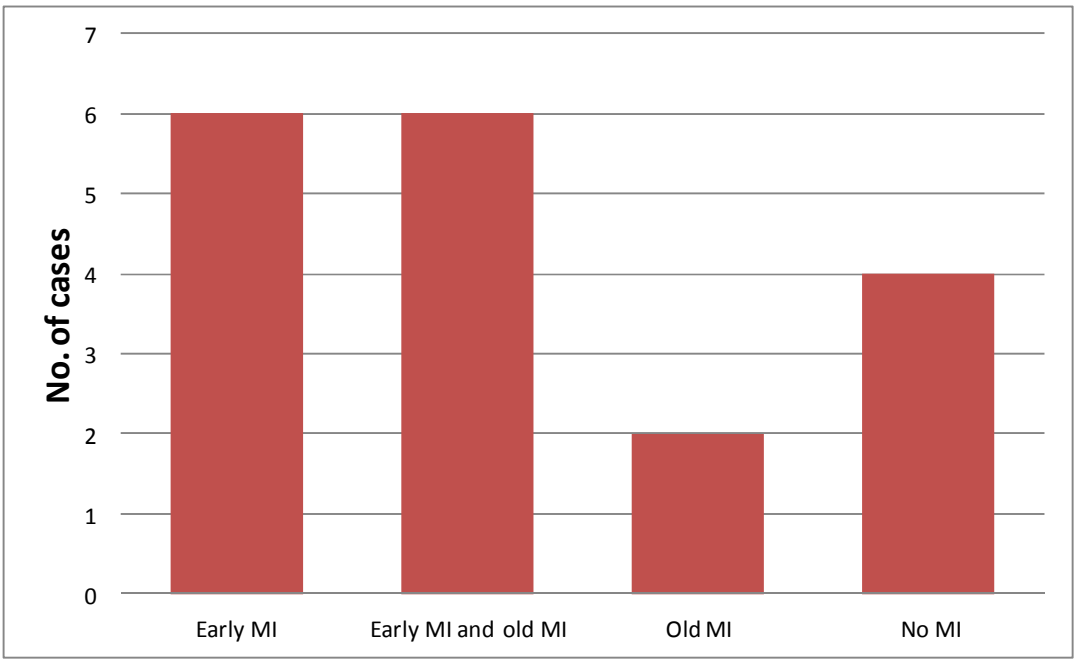
S.No	OBSERVATION	No. OF CASES	INFERENCE
1	Unstained Area of the myocardial tissue	6	TTC Positive for early MI
2	Areas of unstaining of the myocardium and areas of unstaining due to old infarct	6	TTC Positive for early MI and old MI
3	No unstaining of the myocardium except for unstaining due to old fibrotic infarct	2	TTC Negative for early MI and positive for old MI
4	All area of the myocardium stained and no unstained area.	4	TTC Negative for both MI



HISTOPATHOLOGICAL EXAMINATION RESULTS:

S.No	OBSERVATIONS	No. OF CASES
1	Evidence of early myocardial infarction	**6**
2	Evidence of early myocardial infarction and old infarction	**6**
3	Evidence of old infarction	**2**
4	Normal myocardial histology. No areas of infarction	**4**

HPE RESULT OF THE TTC STAINED HEART SLICE CHART



Histopathological examination of the TTC stained heart slices for early myocardial infarction showed features of myonecrosis, waviness of fibers, nucleomegaly, Pyknotic nuclei and polymorphs infiltrations. In areas of old infarction, Histopathological examination revealed evidence of dense fibrous tissue replacing normal myocardial tissue. In HPE positive for early infarction heart, not all the above said features of early myocardial infarction were present. Presence of any one of the features of

the early myocardial infarction was taken into consideration for positive HPE for early myocardial infarction.

Table: 5

COMBINED RESULTS OF TTC STAINING METHOD AND HPE

POSITIVE/ NEGATIVE	HPE Positive for Early MI	HPE Positive for Early MI and Old MI	HPE Negative for Early MI and Positive for Old MI	HPE Negative for both MI
TTC Positive for Early MI	**5**	**0**	**0**	**1**
TTC Positive for Early MI and Old MI	**0**	**6**	**0**	**0**
TTC Negative for Early MI and Positive for Old MI	**0**	**0**	**2**	**0**
TTC Negative MI	**1**	**0**	**0**	**3**

Out of 18 hearts stained using 1%Triphenyl Tetrazolium Chloride solution, 6 hearts with no apparent gross change showed areas of

unstaining were considered TTC positive for early myocardial, Histopathological examination of the 6 TTC positive hearts showed features of early myocardial infarction in 5 hearts, One TTC positive for early infarction heart showed features of normal myocardium i.e. Considered as false TTC positive.

5 hearts showed areas of old infarct, one heart showed area of old infarct and area of hyperemia with softening on gross examination. TTC staining of these 6 hearts showed area of unstaining in addition to the area of unstaining due old infarct (which is evident on gross examination) and were considered TTC positive for early myocardial infarction and old infarction. Histopathological examination of the 6 TTC positive hearts showed features of both early myocardial infarction and features of old infarction.

In two cases there is evidence of old infarct scar on gross examination which remain unstained and the other area were stained completely. Histopathological examination of this unstained old infarct revealed features of old infarction and stained area of the same slice revealed normal histology.

In 4 cases the entire heart slice stained (no unstaining area) and considered TTC negative for early and old infarction. Histopathological

examination revealed features of normal myocardial histology in 3 cases. In one TTC negative heart, Histopathological examination revealed features of early myocardial infarction and considered as false TTC negative.

TTC STAINING AND HISTO PATHOLOGICAL EXAMINATION CONFIRMED CASES

Positive / Negative	Positive for Early Myocardial Infarction by Histopathological examination	Negative for Early Myocardial Infarction by Histopathological examination
TTC Positive for early MI	11	1
TTC Negative for early MI	1	5

The sensivity of the test is 91.66%

The specificity of the test is 83.33%

The positive predictive value of the test is 91.66%

The negative predictive value of the test is 83.33%.

Overall Diagnostic validity of the test is 88.88%

Likelihood ratio for positive test: 5.49.

Likelihood ratio for negative result: 10

DISCUSSION

Discussion

Sudden cardiac death due acute myocardial infarction is common among both men and women. WHO projects, by the year 2020, ischemic heart disease ranks one among the disease burden. In many cases death occurs rapidly and evidence of acute MI is not detected during postmortem examination. Apparent gross morphological changes of acute myocardial infarction take 24 to 48 hrs for its appearance. Usually, diagnosis of myocardial infarction is made by random sectioning of the heart for Histopathological examination. Random sectioning of heart for Histopathological examination is inefficient, as often the method may miss myocardial infarction if the section does not include the inapparent infarct area.

To overcome the difficulty Forensic pathologists have adopted various methods to establish diagnosis of acute myocardial infarction. Triphenyl Tetrazolium chloride staining of the heart for early myocardial infarction is a simpler, easier, rapid technique to carry in autopsy hall and it is possible to provide fair result at the end of the autopsy. Many animal studies conducted using TTC for detection of acute myocardial infarction, may not be applicable directly to humans due to species difference.

The reasons for false positive result are heart slice remaining in contact with the container during incubation in the TTC solution, prolonged exposure of TTC solution to light, old TTC solution, contamination of the solution and greater postmortem interval resulting in autolysis.

Adegboyega et al in 1997 reported that Histochemical staining using TTC for gross detection of early infarction has diagnostic sensivity of 77.4% and specificity of 92.6%. The positive predictive value of the test was 80.5% and negative predictive value was 91.2%. The overall efficiency of the test was 88%[1]

M Shankar Bakkannavar et al in 2011, Histochemical staining using TTC for detection of acute myocardial infarction was conducted on 40 hearts removed from cases of sudden death. The study showed that sensitivity and specificity of the TTC staining method is 100%. In the study, hydrochloric acid is used for adjustment of pH of the solution instead of phosphate buffer[34].

There are various factors which affects the outcome of the experiment.

1) Postmortem interval which strongly affects the activity of the enzymes. In the present study most of the cases are brought dead to casualty and the exact time of death is not known to either the

investigating officers or the relatives. Therefore the Postmortem interval given in the Master chart is only the approximate time since death derived from the information furnished by the investigating officer. In general greater the postmortem interval higher will be the inactivation of dehydrogenase enzymes. There is a rapid decrease in enzyme content in the first 6 hrs of postmortem interval when the body is stored at 37^{o} c. In heart stored at 4^{o}c, the enzyme activity is not altered significantly even the body is stored as long as 6 days.[1]

2) Photosensivity of the Tetrazolium dye: Prolonged exposure of TTC or TTC solution to light leads to inactivation of the tetrazolium salt resulting in false positive outcome. To prevent light exposure the salt or the solution is stored in amber colored bottle which in turn covered with black plastic paper.

3) Storage: Prolong storage decreases the efficacy of the dye in identifying myocardial infarction. Reconstituted TTC solution older than 3 months was not used[1].

4) pH of the solution: pH of the TTC solution is much more important than the concentration of TTC in the solution. TTC solution works well in the range of pH between 7.5 and 9.0, best results are obtained at 7.8[1].

The inability to demonstrate an acute infarction in sudden cardiac death is not due to technical problem, but occurs because in some cases death occurs due to arrhythmias during ischemic period before the development of an infarct.[13]

Limitations of the TTC Histochemical method in diagnosing early myocardial infarction are

1. The test cannot be applied for decomposed cases, as autolysis of the myocardial tissue may produce false positive result.

2. If patient dies of arrhythmias during the ischemic period before the development of infarction, loss of cell wall integrity and intracellular dehydrogenase enzymes will not occur. TTC staining method in such cases will not be helpful in finding out cause of death.

3. Patients with old infarct scar in the heart are at risk of developing ventricular tachy arrhythmias. The mechanism of ventricular tachy arrhythmias is reentry involving the infarct scar, particularly the border areas or other scar areas with deranged conduction[5]. In such cases, TTC method has its limitation.

CONCLUSIONS

Conclusions

The present study concludes that Histochemical staining of heart using Triphenyl Tetrazolium Chloride is a reliable method in the detection of early myocardial infarction for Forensic pathologist during postmortem examination, as the method in the present study has got the diagnostic validity of 88.8%. The preparation of 1% TTC solution, adjustment of pH using low pH and high pH phosphate buffer is quite easy, staining method is cost effective, simple to carry out in autopsy hall and does not require any complex equipments.

The method not only helps Forensic Pathologist in the identification of early myocardial infarction, to a large extent it helps the General Pathologist to know where the sectioning has to be made out for diagnosing early myocardial infarction. However, if death occurs due to arrhythmias during the ischemic period before the development of infarction, the method has limitation in the identification of cause of death due to arrhythmias. In such situation, present and past history of the patient, circumstances, the ultra structural examination of myocardial tissue and conducting fibers helps in identification of the cause of death.

As there is an occurrence of false positive and false negative result in Histochemical staining using TTC for early myocardial infarction,

combination of both Histochemical technique and Histopathological examination helps in diagnosing or ruling out inapparent early myocardial infarction as cause of death in a far number of cases.

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INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No: 04425305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To
Dr. Thunder Chief .K
PG in MD Forensic Medicine
Madras Medical College , Ch-3

Dear Dr. Thunder Chief. K

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled " Diagnosis of early myocardial infarction by histochemical staining of heart on autopsy table" No. 26012011.

The following members of Ethics Committee were present in the meeting held on 28.01.2011 conducted at Madras Medical College, Chennai -3.

1. Prof. S.K. Rajan, MD	– Chairperson
2. Prof. A. Sundaram, MD Dean i/c , Madras Medical College, Chennai -3	– Member Secretary
3. Prof R. Sathianathan Director , Institute of Psychiatry, MMC,Ch-3	– Member
4. Prof R. Nandhini, MD Director, Institute of Pharmacology, MMC, Ch-3	– Member
5. Prof. Geetha Subramanian, MD,DM Prof. & Head , Dept. of Cardiology, MMC, Ch-3	– Member
6. Prof. Md. Ali, MD, DM Professor & Head ,,Dept. of MGE, MMC, Ch-3	– Member
7. Thiru. T.S. Bharathidasan Administrative Officer, MMC, Chennai -3	– Layperson
8. Thiru. S. Govindasamy . BA.BL	– Lawyer
9. Tmt. Arnold Soulina	– Social Scientist

We approve the Proposal to be conducted in its presented form.

Sd /. Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report

Member Secretary, Ethics Committee

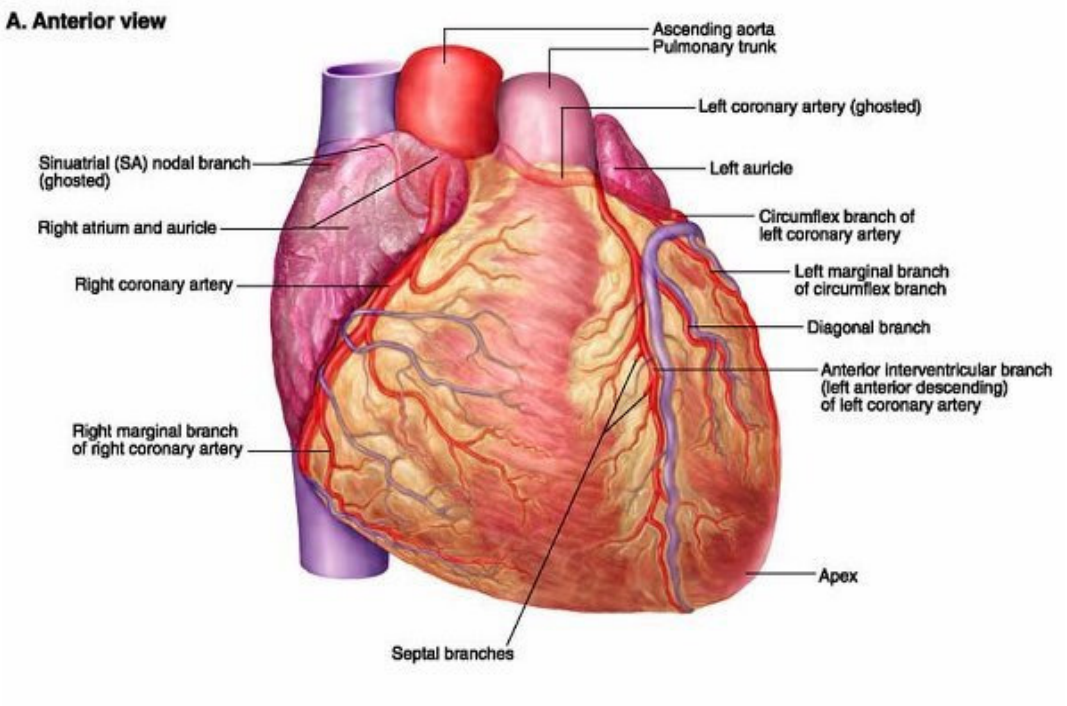

Fig .1 Anterior view of heart showing the anatomy of coronary arteries

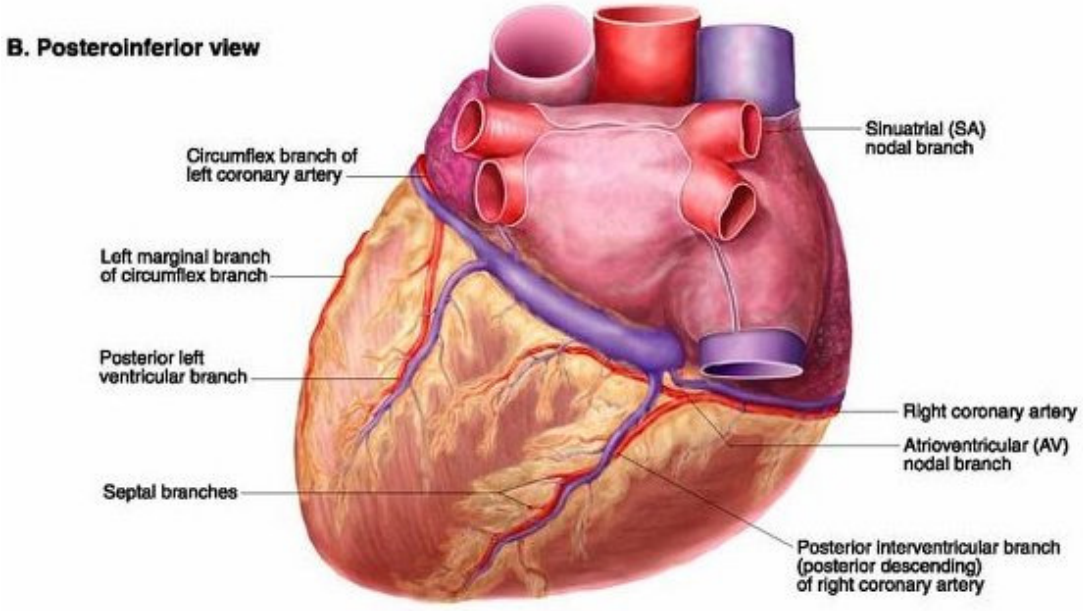

Fig.2 Posterior view of heart showing distribution of coronary arteries

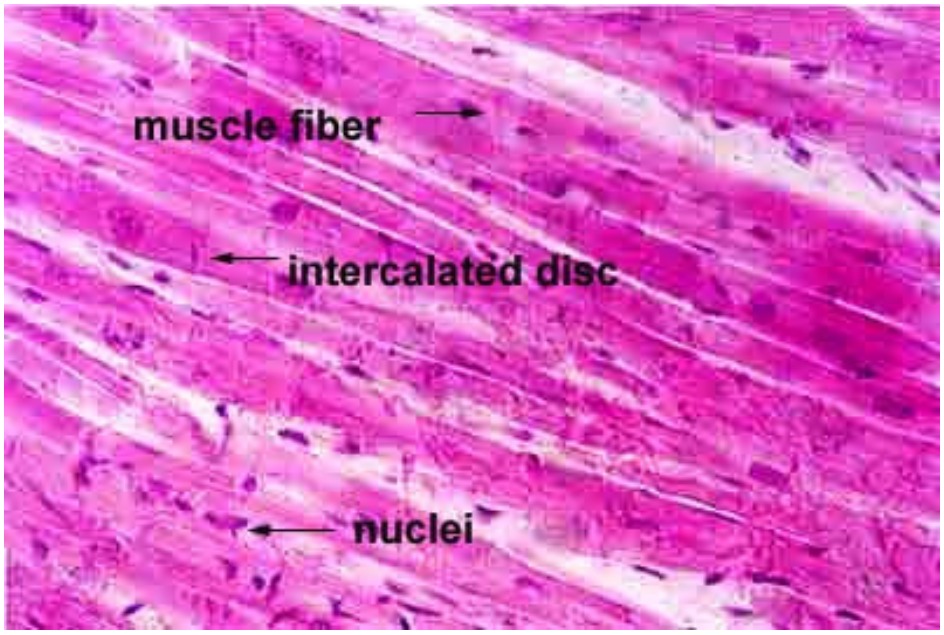

Fig.3 Microanatomy of normal myocardial tissue.

Fig 4. High pH phosphate buffer, Di Sodium hydrogen Phosphate .

Fig.5 Low PH phosphate buffer, Sodium Dihydrogen Phosphate.

Fig.6 Distilled water used for preparation of TCC solution

Fig .7 Tetrazolium Dye (2,3,5 Triphenyl Tetrazolium Chloride - TTC)

Fig.8 Amber color bottle for storing 1% TTC solution.

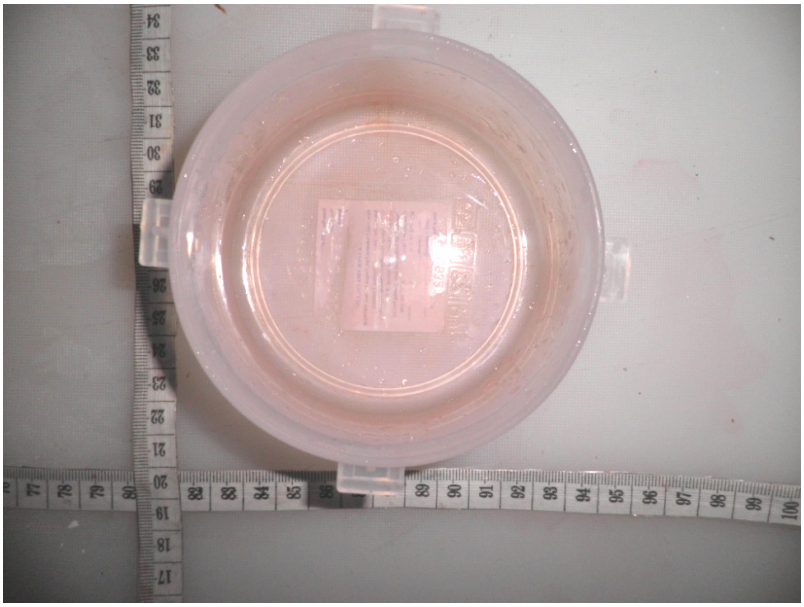

Fig.9 Container for incubating Heart slice in TTC solution

Fig .10 Heart slice before staining.

Fig.11 Heart slice after staining, all areas of the ventricle stained

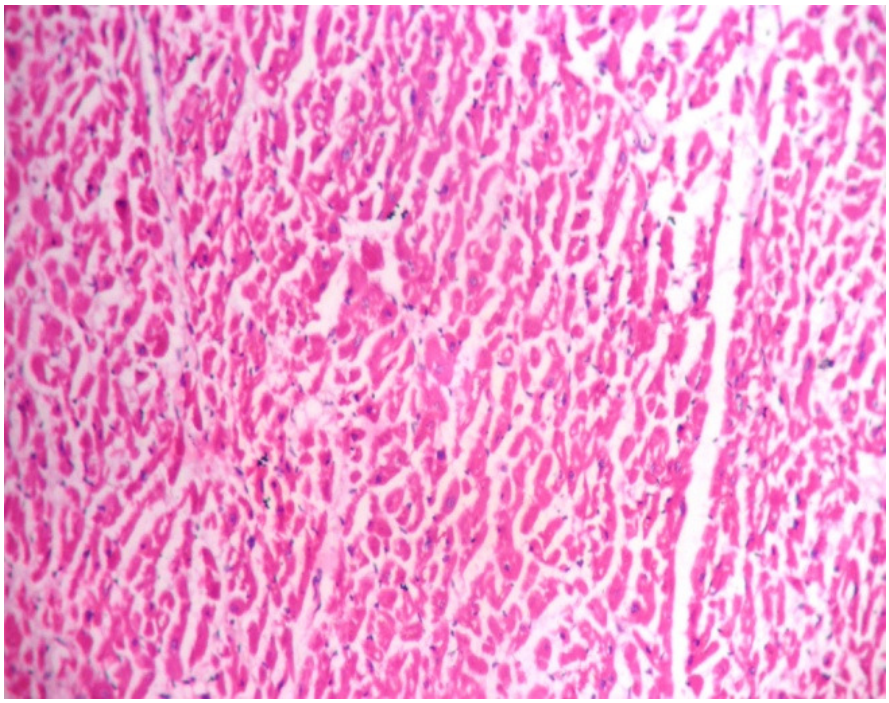

Fig.12 Micro Photo 10X Transverse section histology shows Normal myocardium

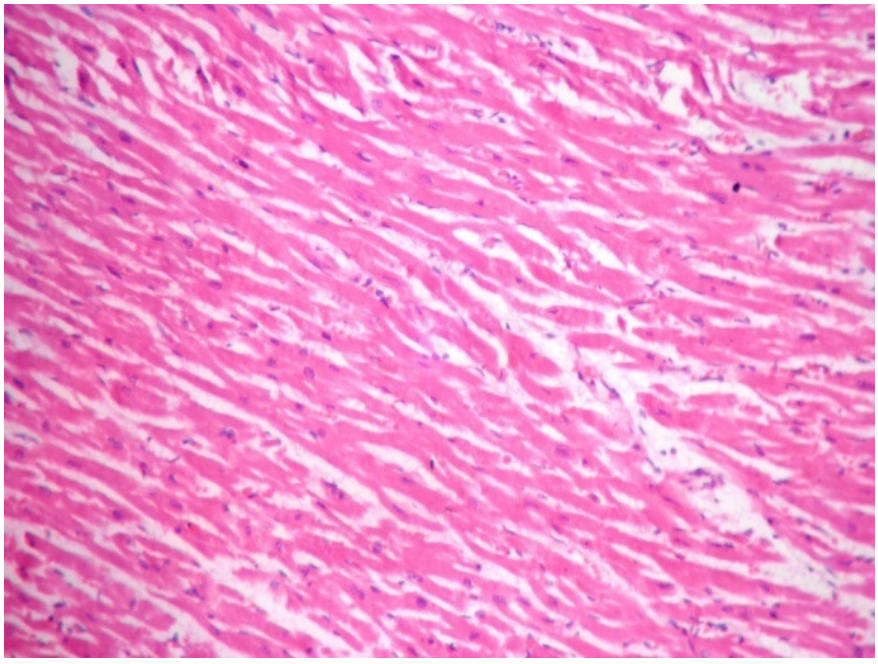

Fig.13 Micro Photo 10X Longitudinal section shows myocardial fibers.

Fig.14 Heart slice before staining.

Fig.15 Heart slice after staining, shows unstaining of antero lateral wall and adjoining IV septum

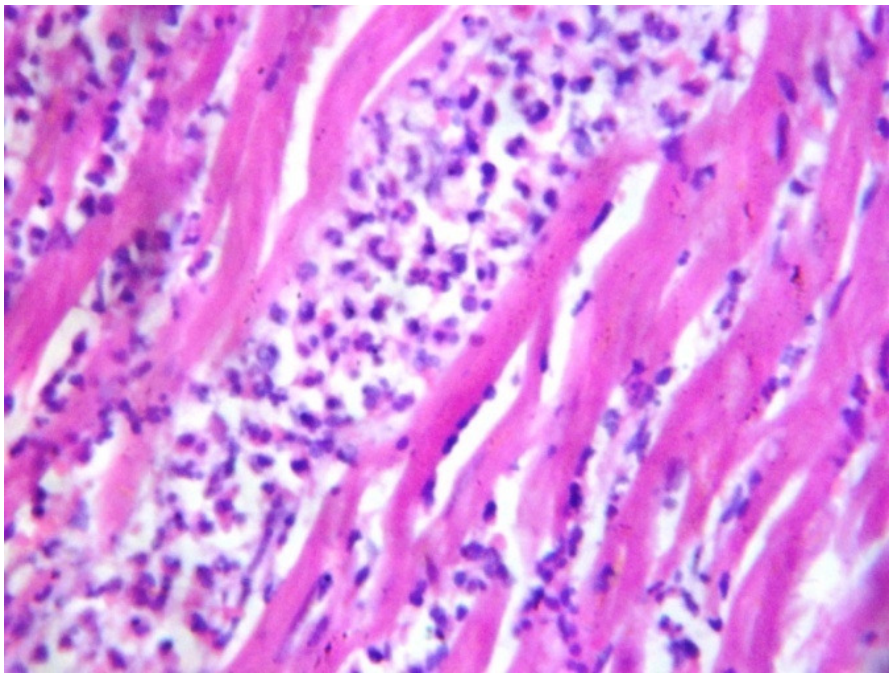

Fig.16 Micro photo 40X shows infiltration of neutrophils Infiltration

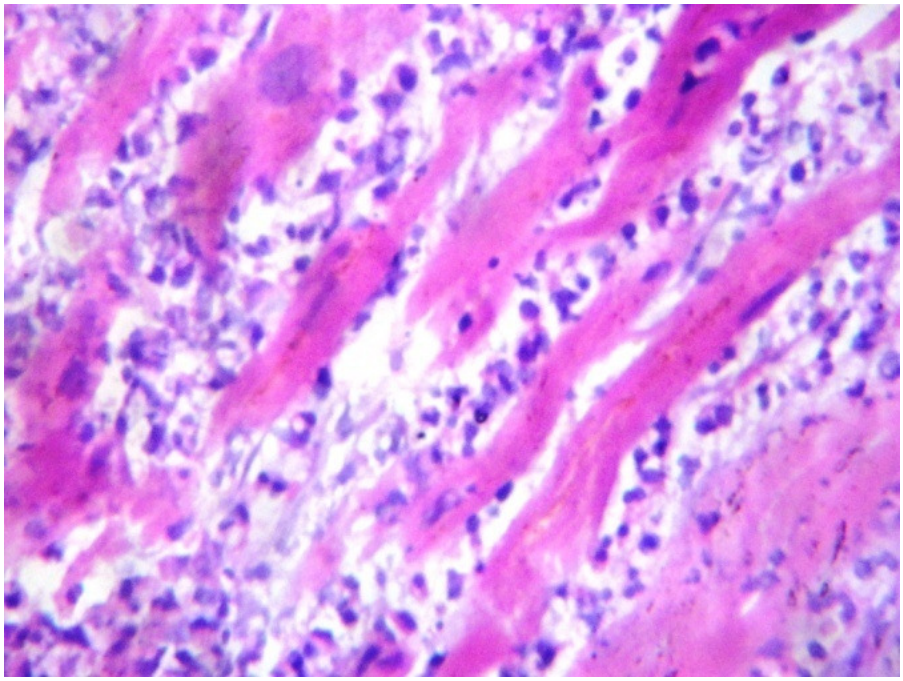

Fig.17 Micro Photo 40X shows Neutrophils infiltration, Karyorrhexis, karyomegaly, Pyknotic nuclei and Myonecrosis.

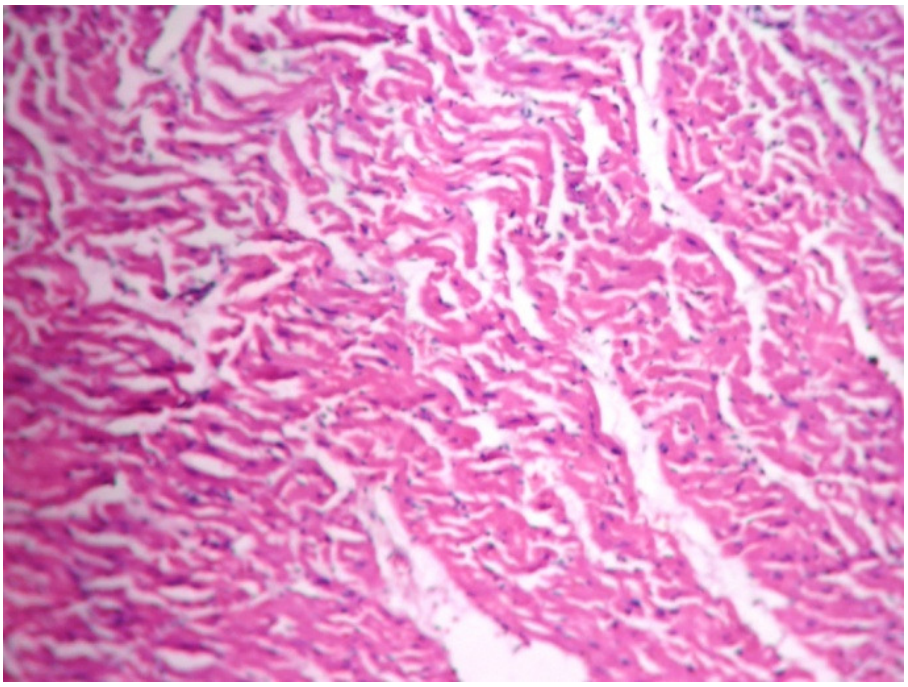

Fig.18 Micro Photo 10X shows Waviness of Myocardial fibers.

Fig. 19 Heart slice before TTC staining shows old infarct fibrous scar in the posterior wall of left ventricle

Fig.20 Heart slice after TTC staining shows unstaining of posterior wall and sub endocardial region of the lateral wall of the left vent

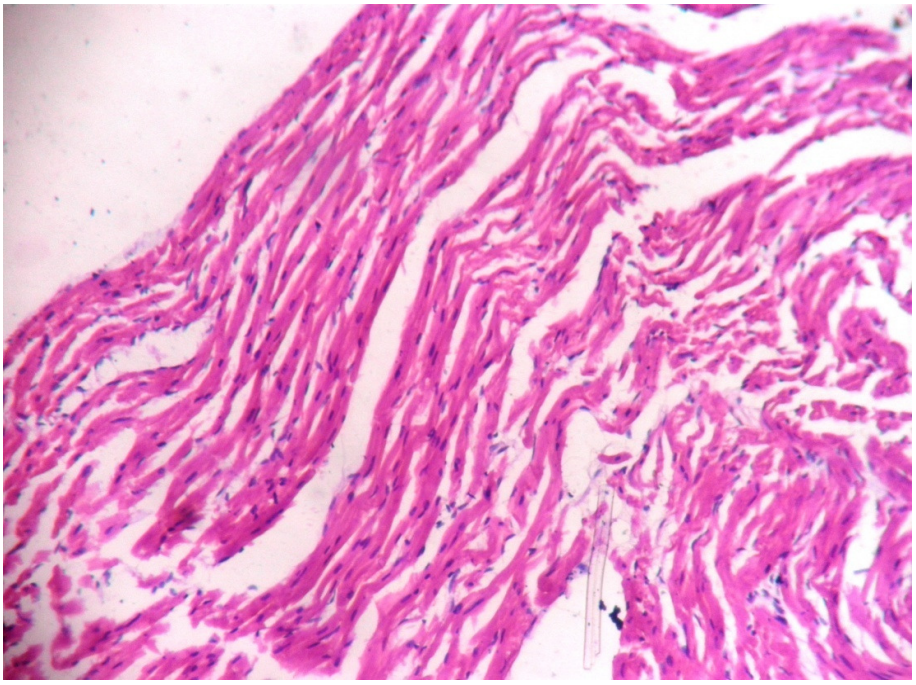

Fig.21 Micro Photo 10X shows waviness of myocardial fibers.

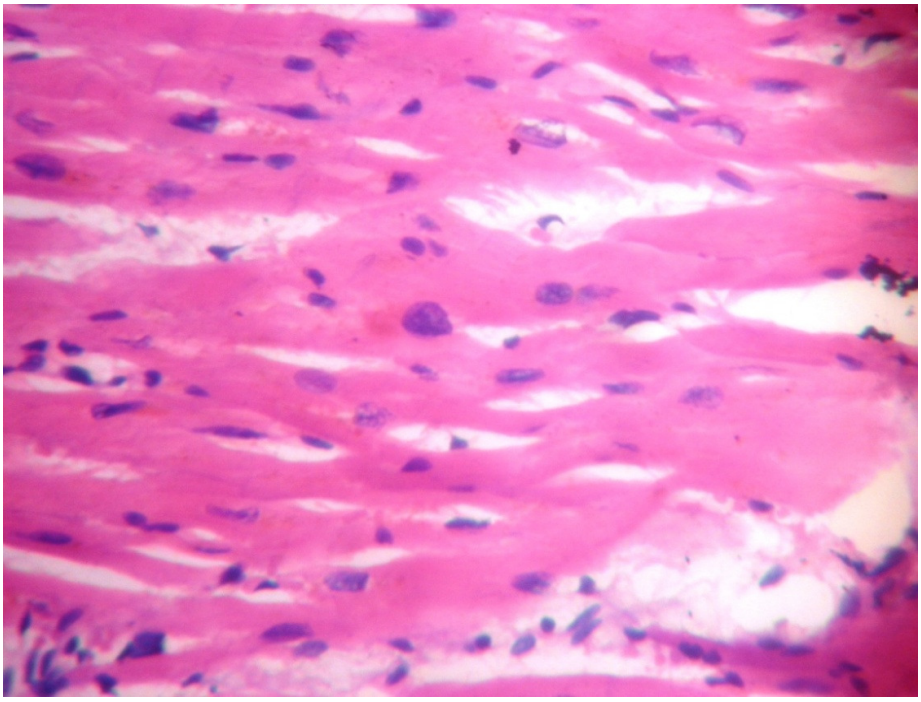

Fig .22 Micro Photo 40X shows Pyknotic Nuclei

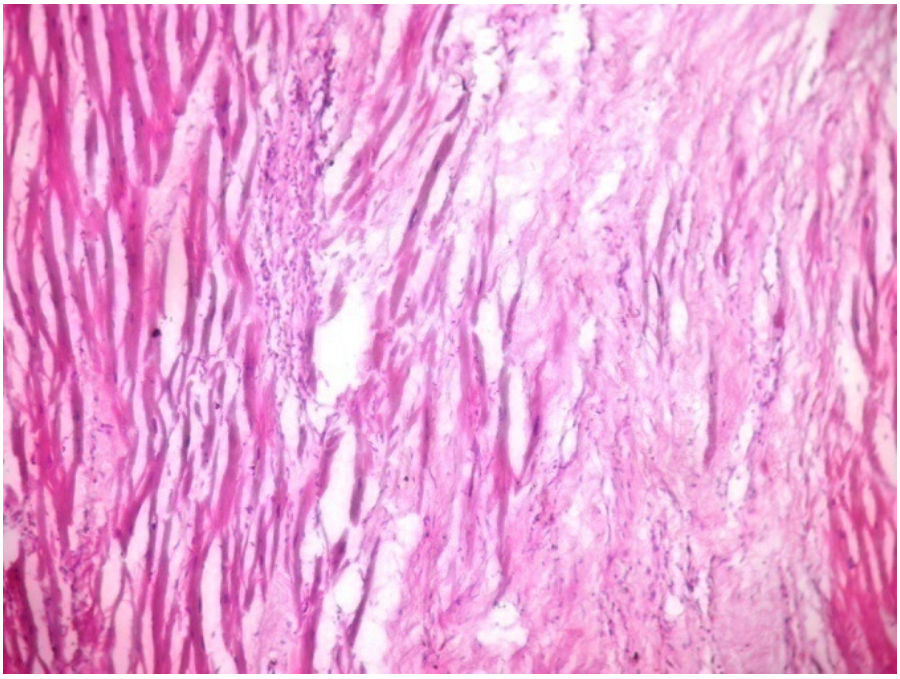

Fig.22 Micro photo 10 X shows old dense fibrous tissue and adjoining normal myocardium

TITLE: DIAGNOSIS OF EARLY MYOCARDIAL INFARCTION BY HISTOCHEMICAL STAINING OF HEART ON AUTOPSY TABLE

Author: K. Thunder Chief,

Co Author: Capt. Dr B. Santhakumar M.Sc., MD., DipNB(FM), P.G.D.M.L.E,

Institute of Forensic medicine, Madras Medical College, Chennai-3

INTRODUCTION:

The incidence of Sudden Unexpected Death is increasing globally. Cardio vascular disease is the most common cause for sudden death. Eighty percent of sudden cardiac death is due to coronary arterial disease[36]. In 25% of cases death occurs abruptly and unexpectedly within first hour of onset of clinical symptom. Most often, the identification of early change of Myocardial infarction becomes difficult during postmortem examination, as gross change of infarct will not be apparent for 24 to 48 hrs following myocardial ischemic damage Histochemical staining techniques using azo dyes are based on the fact that ischemic myocardial cells lose their membrane integrity and release their enzyme contents in to the blood, resulting in a marked decrease or total depletion of these enzymes in the ischemic areas of the myocardium. Enzyme depleted infarct myocardium remain unstained, which is the principle of this study.

AIMS AND OBJECTIVES:

- To diagnose early Myocardial Infarction by Histochemical staining of Myocardium using Triphenyl Tetrazolium Chloride (TTC) while performing autopsy, in the absence of appreciable macroscopic changes in the myocardial tissue.
- To confirm those identified areas of early myocardial infarction by Histopathological Examination.
- To determine the diagnostic validity of the Histochemical Staining (TTC) of heart in gross detection of early Myocardial Infarction.

MATERIALS AND METHODS:

Slices from 18 hearts of suggested or suspected sudden cardiac death were incubated in 1% Triphenyl Tetrazolium Chloride Solution for 20 min. after

adjusting the pH of the solution using phosphate buffer. The heart slices were examined for unstained area. Normal myocardium was stained whereas infarct areas remain unstained. Both stained and unstained areas were subjected to Histopathological examination for early Myocardial Infarction.

OBSERVATION AND RESULTS:

Of 18 hearts stained with TTC solution, 11 hearts showed positive TTC result, 5 hearts showed negative result, 1 heart showed false positive result and 1 heart showed false negative result.

CONCLUSION:

Histochemical staining using 1% TTC solution is a reliable method in detection of early myocardial infarction. Though this method has diagnostic validity of 88.88%, it could not identify death due to arrhythmias.